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Piceatannol Enhances Intracellular Energy Metabolism via SIRT1 in C2C12 Cells

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Abstract

Skeletal muscle is the largest organ in the human body, and improving energy metabolism is crucial for maintaining and promoting health, including alleviating metabolic disorders like obesity and diabetes. Piceatannol (PIC), a polyphenol in passion fruit seeds, has various beneficial effects, including blood glucose regulation, vasodilation, and antitumor activities. Although PIC has been reported to induce SIRT1 expression—a key regulator of energy metabolism—in skeletal muscle, its actual impact on metabolic function remains largely unclear. Therefore, in this study, we comprehensively analyzed the effects of PIC on energy metabolism in skeletal muscle cells. By adding PIC to C2C12 myotubes, we examined the expression of multiple metabolism-related genes and proteins—such as SIRT1, PGC1A, PDK4, and PPARG—using real-time PCR and Western blotting techniques and measured mitochondrial activity using a flux analyzer, confirming that PIC enhances energy metabolism in skeletal muscle. Additionally, we observed an increase in intracellular NAD^+ levels, activation of the NAD metabolic pathway, and enhanced lipid metabolism. Furthermore, when SIRT1 expression was suppressed using siRNA, the metabolic enhancement induced by PIC was abolished, demonstrating that SIRT1 plays a central role in PIC-induced activation of skeletal muscle energy metabolism.

Keywords

Skeletal Muscle, Piceatannol, Energy Metabolism, SIRT1, Mitochondria

1. Introduction

Skeletal muscle is the largest organ in the human body, accounting for about 40% of total body weight in non-obese individuals. Forearm oxygen uptake, an indica-

tor of endurance capacity, constitutes about 20% - 30% of total oxygen consumption [1]. Skeletal muscle is involved in glucose uptake and glycogen synthesis and is responsible for approximately 80% of postprandial glucose disposal [2]. During exercise, the uptake and oxidation of long-chain fatty acids increase, allowing lipids to serve as an energy source [3]. Numerous studies have demonstrated the involvement of skeletal muscle in whole-body energy metabolism, such as the association between impaired muscle mitochondrial function and insulin resistance [4] and the reduction in skeletal muscle glucose uptake in patients with type 2 diabetes [5]. These findings indicate that improving energy metabolism in skeletal muscle is crucial not only for addressing the increasing prevalence of metabolic disorders like obesity and diabetes, but also for maintaining and enhancing health and quality of life.

Peroxisome proliferator-activated receptor γ coactivator-1α (PGC1A) interacts with various transcription factors involved in thermogenesis, mitochondrial biogenesis, glucose metabolism, and lipid metabolism [6], playing a central role in energy metabolism. Increased PGC1A expression in skeletal muscle promotes mitochondrial biogenesis and fatty acid utilization, contributing to improved exercise capacity [7]. PGC1A expression is regulated upstream by sirtuin 1 (SIRT1). Sirtuins are NAD^+-dependent histone deacetylases that help activate energy metabolism [8]. SIRT1, mainly located in the nucleus, is regulated by changes in the NAD^+/NADH ratio and modulates gene expression in response to intracellular metabolic changes [9]. In skeletal muscle, SIRT1 induces fatty acid oxidation during nutrient deprivation through the deacetylation of PGC1A, regulates muscle differentiation [10] [11], and improves insulin sensitivity via enhanced mitochondrial function [12]. Thus, SIRT1 is a key regulator of gene expression in skeletal muscle, responding to various metabolic states.

Similar to PGC1A, SIRT1 also controls the activity of peroxisome proliferator-activated receptors (PPARs), which are nuclear receptors. There are three PPAR isoforms: PPARα (PPARA), PPARβ/δ (PPARB/D), and PPARγ (PPARG) [13]. In skeletal muscle, PPARA and PPARB/D regulate free fatty acid (FFA) uptake and fatty acid oxidation [14], while PPARG is involved in reducing lipid accumulation through enhanced FFA oxidation [15] and improving glucose uptake and insulin sensitivity [16]. During exercise, PPARB/D prevents the ubiquitination of PGC1A, thereby increasing mitochondrial energy metabolism [16]. Furthermore, pyruvate dehydrogenase kinase (PDK) 4 is a gene regulated by PGC1A and PPARs, which are substrates of SIRT1 [16] [17]. PDK4, one of the PDK isoenzymes, is primarily expressed in skeletal muscle and heart and negatively regulates glucose oxidation. In skeletal muscle, PDK4 activation has been observed under the forced expression of PGC1A and upon administration of PPARA agonists [17] [18].

Recently, polyphenols have gained attention for their role in enhancing energy metabolism. Polyphenols such as resveratrol, found in wine and grape skins, quercetin in onions, and catechins in tea, have been reported to suppress oleic acid-induced fat accumulation in hepatocytes, protect mitochondrial membrane po-

tential, and regulate the expression of fatty acid oxidation-related enzyme genes. These polyphenols enhance fatty acid β-oxidation via SIRT1 activation [19]. Among the polyphenols that suppress oleic acid-induced fat accumulation and promote lipid metabolism is piceatannol (PIC), abundant in passion fruit seeds [20] [21]. PIC, part of the stilbene family, exhibits stronger antioxidant activity than many other polyphenols [20] [22]. Its antioxidant properties suppress reactive oxygen species (ROS) generated by ultraviolet rays, inhibiting photoaging [23], and promote hyaluronic acid synthesis, strengthening skin structure and function via SIRT1 induction [24]. In skeletal muscle, PIC protects cells from oxidative stress by increasing the expression of antioxidant factors such as heme oxygenase-1 (HO-1) and superoxide dismutase (SOD1) [22], and promotes glucose uptake by facilitating GLUT4 translocation through AMPK activation [25]. Recent studies suggest PIC's involvement in skeletal muscle energy metabolism, including increased expression of SIRT1 and mitochondrial metabolism-related genes upon PIC treatment of C2C12 cells [21].

However, the precise mechanism by which PIC enhances energy metabolism in muscle remains unclear. Therefore, in this study, we investigated the effect of PIC on muscle energy metabolism in C2C12 cells.

2. Materials and Methods

2.1. Cell Culture and Treatments

Mouse-derived C2C12 myoblasts were cultured in Dulbecco's modified eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 1% penicillin-streptomycin and 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA) at 37°C and 5% CO_2. To induce differentiation into myotubes, cells were grown in growth medium until confluence and then switched to differentiation medium [DMEM containing 2% horse serum (HS; Gibco)] for 4 days. After treatment with either 0.1% dimethyl sulfoxide (DMSO; Sigma-Aldrich) or 100 μM PIC (Tokyo Chemical Industry, Tokyo, Japan), cells were washed twice with PBS and collected in ISOGEN (NIPPON GENE, Tokyo, Japan) or lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM NaF, 2 mM $NaVO_4$, 1 μM MG132].

2.2. Cellular Metabolic Rate

Intracellular energy metabolism in C2C12 myotubes was evaluated using a Seahorse X24 Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA). On day 4 of differentiation, cells were treated with DMSO or 100 μM PIC for 6 hours, then subjected to time-course measurements in Seahorse X24 plates. Oxygen consumption rates (OCRs) were measured three times at baseline and after the sequential injection of 2.5 μM oligomycin at 19 minutes (Sigma-Aldrich), 2.5 μM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP; Sigma-Aldrich) at 46 minutes, and 2.5 μM rotenone (Sigma-Aldrich) plus 2.5 μM antimycin (Sigma-Aldrich) at 73 minutes. Basal respiration, mitochondrial proton leak, maximal respiration, and

non-mitochondrial respiration values were determined according to the Seahorse user manual.

2.3. ATP Assay

On day 4 of differentiation, C2C12 cells were treated with DMSO, 100 μM, or 200 μM PIC for 48 hours. After each treatment, intracellular ATP levels were measured using the ATP Assay Kit-Luminescence (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Luminescence was determined using a microplate reader (SpectraMax i3; Molecular Devices, San Jose, CA, USA).

2.4. Quantitative Real-Time PCR (RT-PCR) Analysis

C2C12 myotubes treated with DMSO, 50, or 100 μM PIC for 24 hours were collected in ISOGEN (NIPPON GENE, Tokyo, Japan). Chloroform (Nacalai Tesque, Kyoto, Japan) was added with vigorous mixing. The samples were centrifuged at 12,000 rpm for 15 minutes at 4°C, and the supernatant was purified by isopropanol precipitation followed by 80% ethanol precipitation. Using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan), purified RNA (1000 μg/μl) was reverse-transcribed into cDNA. Real-time PCR was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primer sequences used are listed in Table 1. β-actin served as the internal control.

Table 1. Primer sequences.

Gene		Sequence (5'-3')
b-actin	F	CATCCGTAAAGACCTCTATG
	R	ATGGAGCCACCGATCCACA
SIRT1	F	GACGCTGTGGCAGATTGTTA
	R	GGAATCCCACAGGAGACAGA
PDK4	F	AAAGGACAGGATGGAAGGAATCA
	R	TTTTCCTCTGGGTTTGCACAT
PGC1A	F	CGGAAATCATATCCAACCAG
	R	TGAGGACCGCTAGCAAGTTTG
PPARG	F	ATGGAGCCTAAGTTTGAGTT
	R	CAGCAGGTTGTCTTGGATGT
NMNAT1	F	TTCAAGGCCTGACAACATCGC
	R	GAGCACCTTCACAGTCTCCACC
NMNAT2	F	CAGTGCGAGAGACCTCATCCC
	R	ACACATGATGAGACGGTGCCG
NAMPT	F	TCGGTTCTGGTGGCGCTTTGCTAC
	R	AAGTTCCCCGCTGGTGTCCTATGT
NADSYN1	F	AGAGCCTTTGTCCAGTTTTG
	R	GTTGTCATCTTGTGCCTGTT
CD36	F	GCCAAGCTATTGCGACATG
	R	CAATGGTTGTCTGGATTCTGG

Continued

CPT1B	F	CTCCGAAAAGCACCAAAACA
	R	CTCCAGCACCCAGATGATTG
MCAD	F	TTGAGTTGACGGAACAGCAG
	R	AGTTTGCACCCCTGTACACC
LCAD	F	TCACCACACAGAATGGGAGA
	R	ACGCTTGCTCTTCCCAAGTA

2.5. Western Blot

C2C12 myotubes treated with DMSO or 100 μM PIC for 24 hours were collected in lysis buffer. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Ten micrograms of protein were denatured in SDS at 95˚C for 5 minutes. The proteins were separated by SDS-PAGE using Extra PAGE One precast gels (Nacalai Tesque) and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA) using a semi-dry blotting system (ATTO, Tokyo, Japan). After blocking with a 4% Block Ace solution (DS Pharma Biomedical, Osaka, Japan) at room temperature for 1 hour, the membranes were incubated overnight at 4˚C with a 1:1000 dilution of primary antibodies against SIRT1 (Cell Signaling Technology, Danvers, MA, USA), PGC1A (Proteintech, Rosemont, IL, USA), PDK4 (Abcam, Cambridge, UK), PPARG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or β-actin (Sigma-Aldrich). After washing, the membranes were incubated for 1 hour at 37˚C with a 1:5000 dilution of anti-rabbit IgG or anti-mouse IgG secondary antibodies (Cell Signaling Technology). Immunoreactive proteins were detected using ImmunoStar (FUJIFILM Wako Pure Chemical, Osaka, Japan) and visualized using a C-Digit chemiluminescent scanner (LI-COR Biosciences, Lincoln, NE) and Image Studio Digits software ver. 5.2.

2.6. SIRT1 Activity Assay

On day 4 of differentiation, C2C12 cells were treated with DMSO or 100 μM PIC for 1, 3, or 24 hours. After each treatment, intracellular SIRT1 activity was measured using the SIRT1 Activity Assay Kit (Abcam), following the manufacturer's instructions. Fluorescence was determined using a microplate reader (Infinite M Nano™; Tecan, Mannedorf, Switzerland).

2.7. NAD Assay

On day 4 of differentiation, C2C12 cells were treated with DMSO or 100 μM PIC for 24 hours. At the end of each treatment, intracellular NAD^+ levels and the NAD/NADH ratio were measured using the NAD/NADH Assay Kit (Dojindo) according to the manufacturer's instructions. Fluorescence was determined using a microplate reader.

2.8. Transfection

On day 4 of differentiation, C2C12 cells were transfected with 200 nM of SIRT1

siRNA or Negative Control siRNA (Thermo Fisher Scientific) using Lipofectamine® RNAiMAX Reagent (Thermo Fisher Scientific), with either DMSO or 100 μM PIC for 24 hours, then harvested using ISOGEN or lysis buffer.

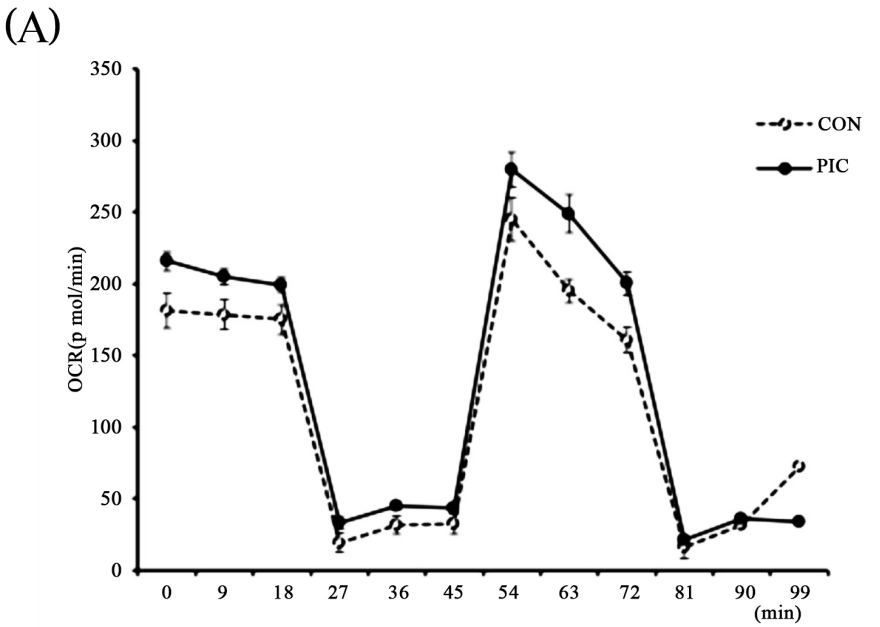
2.9. Statistical Analysis

All data are expressed as the mean ± standard error (mean ± SEM). Ordinary one-way ANOVA was used for statistical analysis, with $p < 0.05$ considered statistically significant. GraphPad Prism Software version 9.3.1 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical processing.

3. Results

3.1. PIC Activated Mitochondrial Respiration

First, to determine whether PIC enhances intracellular energy metabolism in C2C12 cells, we used an extracellular flux analyzer to measure intracellular metabolic dynamics. By sequentially adding inhibitors of mitochondrial respiratory chain complexes, this analyzer determines basal respiration, proton leak, maximal respiration, and spare respiratory capacity. We treated differentiated C2C12 myotubes with 100 μM PIC for 6 hours and then assessed aerobic respiration using the extracellular flux analyzer (**Figure 1(A)**). The PIC-treated group showed a significant increase in basal respiration (**Figure 1(A)**, **Figure 1(B)**) and a trend toward approximately a 20% increase in maximal oxygen consumption compared to the control group (**Figure 1(A)**, **Figure 1(C)**). To investigate changes in energy metabolism induced by PIC, we measured intracellular ATP production. A significant increase in ATP production was observed in myotubes 48 hours after PIC treatment (**Figure 1(D)**). These findings indicate that adding PIC to C2C12 myotubes enhances mitochondrial-driven cellular metabolism via some pathways.



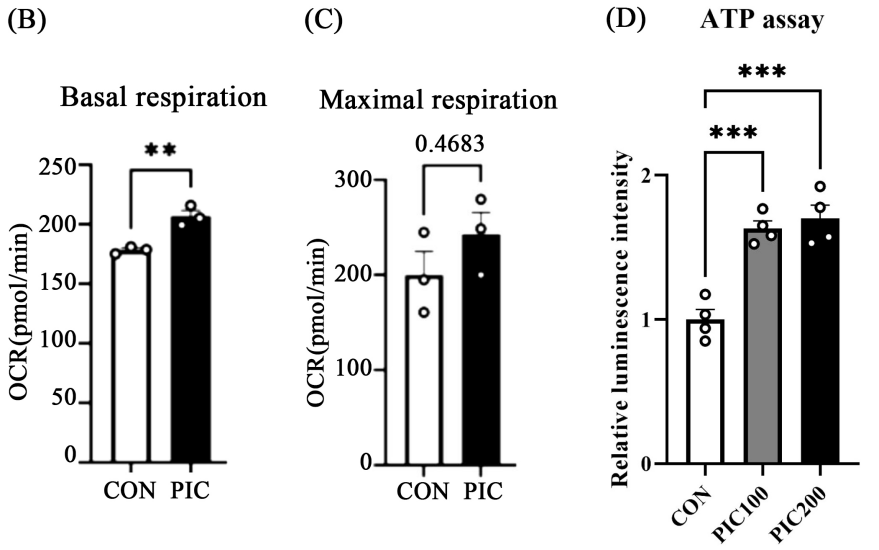


Figure 1. PIC activated mitochondrial respiration. (A) OCR was determined in a Seahorse X24 Flux Analyzer in C2C12 myotubes treated with DMSO or 100 μM PIC for 6 hours under basal, oligomycin (2.5 μM), FCCP (2.5 μM), rotenone (2.5 μM), and antimycin A (2.5 μM) loaded conditions. Results are shown as mean ± SEM (n = 3). (B) Basal respiration and (C) maximal oxygen consumption as derived from (A). Results are shown as mean ± SEM (n = 3). $^{**}P < 0.01$. (D) ATP level was determined in C2C12 myotubes treated with DMSO, 100 μM, or 200 μM PIC for 48 hours. Results are shown as mean ± SEM (n = 4). $^{***}P < 0.001$.

3.2. PIC Enhanced Energy Metabolism-Related Genes and SIRT1 Enzyme Activity

Next, we examined how PIC promotes intracellular metabolism. Given that PIC treatment enhanced mitochondrial activity, we analyzed genes involved in mitochondrial biogenesis and related factors. Previous reports have shown that PGC1A and PDK4 mRNA levels increase in C2C12 cells treated at various concentrations and durations (e.g. 50 μM for 6 hours) [21]. In the present study, we examined these and other metabolic factors under treatment conditions of 100 μM for 24 hours. PIC addition to C2C12 myotubes significantly upregulated both mRNA (**Figure 2(A)**) and protein (**Figure 2(B)**) expression levels of PGC1A and PPARG—both involved in mitochondrial biogenesis—as well as SIRT1, which activates PGC1A through deacetylation, and PDK4, which regulates intracellular lipid metabolism. These results suggest that the increased mitochondrial respiration in skeletal muscle cells induced by PIC is mediated by these energy metabolism-related factors.

Among the genes whose expression increased due to PIC, SIRT1 is noteworthy as it belongs to the sirtuin family of NAD^+-dependent deacetylases, primarily located in the nucleus. SIRT1 is known to regulate several target proteins, including PGC1A, PPARA, PPARG, and the mitochondrial uncoupling protein UCP-2, through deacetylation [26]. Since SIRT1's function depends on both its expression level and enzymatic activity, we evaluated SIRT1 activity following PIC treatment in C2C12 myotubes. We observed a significant increase in SIRT1 activity as early as 1 hour

after PIC addition (Figure 3). These results suggest that PIC controls mitochondrial activity by upregulating energy metabolism-related genes, including SIRT1.

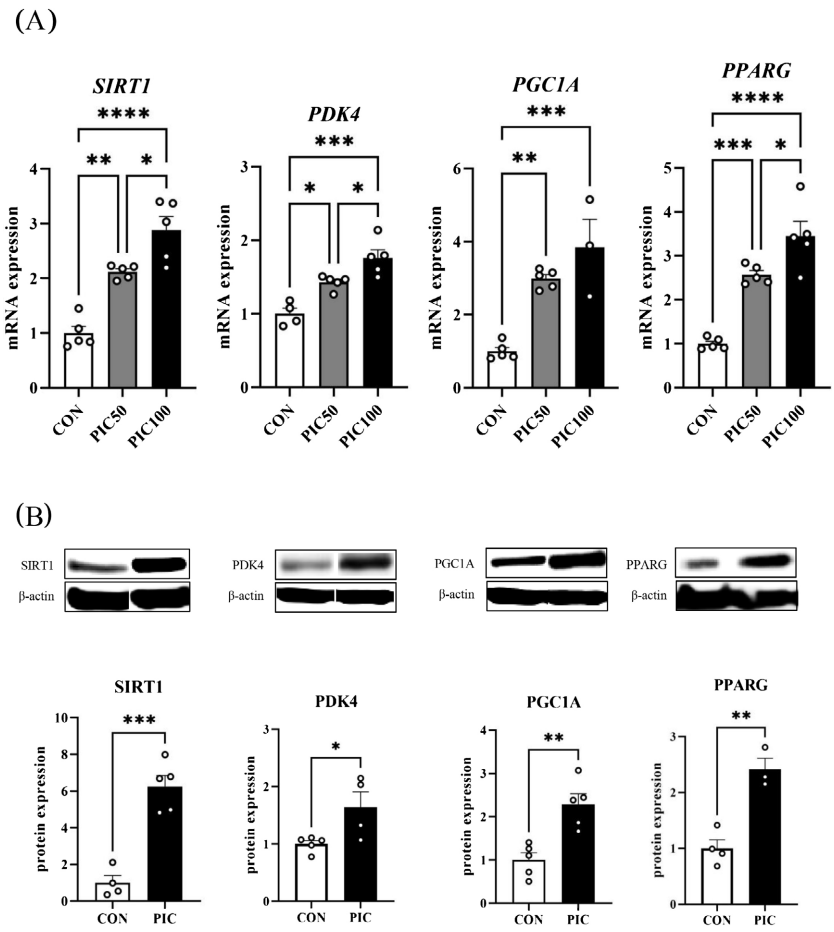


Figure 2. PIC enhanced energy metabolism-related genes protein expression. (A) mRNA expression levels of *SIRT1*, *PDK4*, *PGC1A*, and *PPARG* were determined in C2C12 myotubes treated with DMSO, 50 μM, or 100 μM PIC for 24 hours. Results are shown as mean ± SEM (n = 3 - 5). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. (B) Protein levels of SIRT1, PDK4, PGC1A, and PPARG were determined in C2C12 myotubes treated with DMSO or 100 μM PIC for 24 hours. Results are shown as mean ± SEM (n = 3 - 5). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

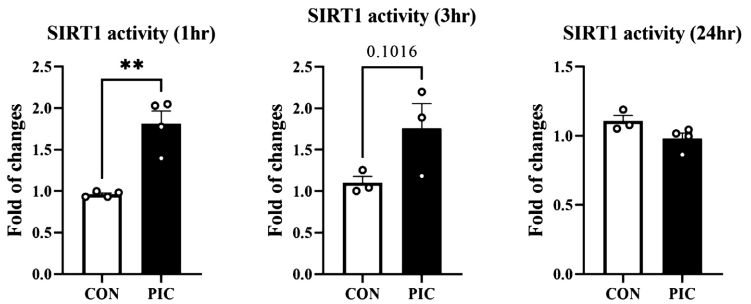


Figure 3. PIC enhanced SIRT1 activity. SIRT1 activity was measured in C2C12 myotubes treated with DMSO or 100 μM PIC for 1, 3, 24 hours. Results are presented as mean ± SEM (n = 3). **$P < 0.01$.

3.3. PIC Activated NAD Metabolism

Among the upregulated energy metabolism-related genes and proteins in response to PIC, SIRT1 was identified as a key player due to its role in regulating other proteins. SIRT1, a deacetylase, consumes NAD^+ to deacetylate substrates. SIRT1 activation is also known to increase intracellular NAD^+ levels [27]. We examined whether PIC treatment affects NAD metabolism in muscle cells. After adding PIC to C2C12 myotubes, we found a slight increase in intracellular NAD^+ levels and a significant increase in the NAD^+/NADH ratio (**Figure 4(A)**). Furthermore, RT-PCR analysis revealed a significant upregulation of NAD^+-synthesis-related genes, including *Nicotinamide mononucleotide adenylyl transferase* (*NMNAT*)1 and 2, *Nicotinamide phosphoribosyl transferase* (*NAMPT*), and *Glutamine-dependent NAD*(+) *synthetase* (*NADSYN*1). These findings indicate that PIC enhances the expression of NAD^+ biosynthetic pathway genes in skeletal muscle cells, thereby increasing intracellular NAD^+ levels (**Figure 4(B)**).

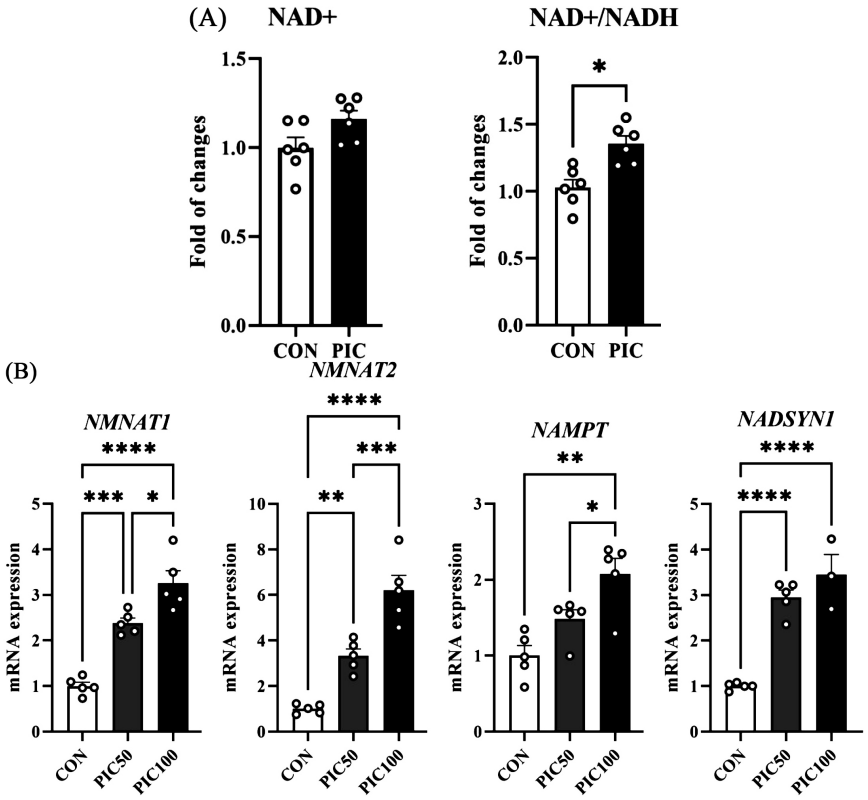


Figure 4. PIC activated NAD metabolism. (A) NAD^+ and NAD^+/NADH ratio were measured in C2C12 myotubes treated with DMSO or 100 μM PIC for 24 hours. Results are presented as mean ± SEM (n = 6). *$P < 0.05$. (B) mRNA expression levels of *NMNAT1*, *NMNAT2*, *NAMPT*, and *NADSYN1* were determined in C2C12 myotubes treated with DMSO, 50 μM, or 100 μM PIC for 24 hours. Results are shown as mean ± SEM (n = 3 - 5). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

3.4. PIC Activated Lipid Metabolism

Energy production via mitochondria is closely linked to intracellular lipid metab-

olism (fatty acid β-oxidation) [28]. Therefore, we analyzed the expression of lipid metabolism-related genes in PIC-treated C2C12 myotubes using RT-PCR. PIC treatment significantly increased the expression of *Carnitine palmitoyl transferase 1B* (*CPT1B*), a key enzyme for long-chain fatty acid transport into mitochondria and β-oxidation; *CD36*, which is responsible for cellular uptake of long-chain fatty acids; and *Medium-chain acyl-CoA dehydrogenase* (*MCAD*) and *long-chain acyl-Coenzyme A dehydrogenase* (*LCAD*), both involved in fatty acid breakdown in the mitochondrial matrix (Figure 5). These results suggest that PIC promotes fatty acid β-oxidation in skeletal muscle cells, thereby increasing the substrates for mitochondrial energy production.

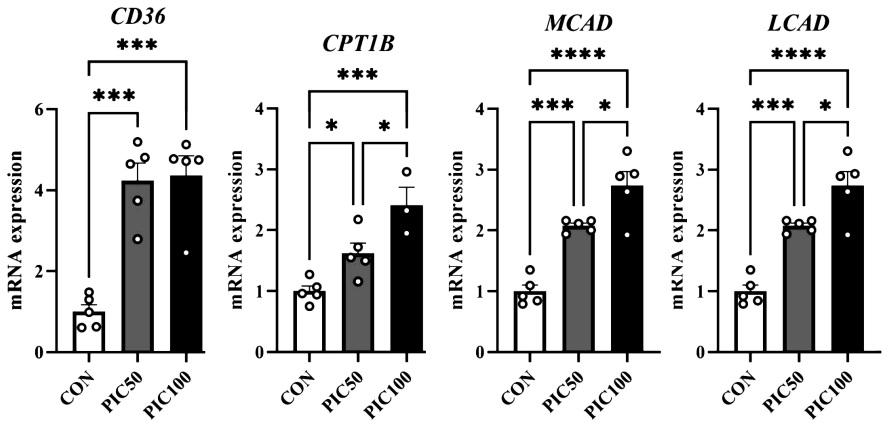
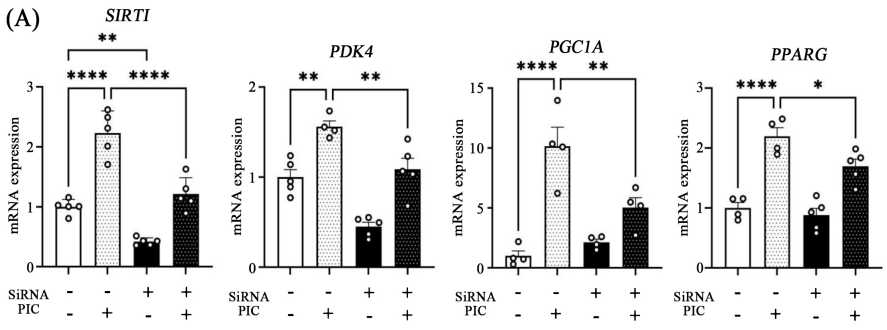


Figure 5. PIC enhanced lipid metabolism-related genes. mRNA expression levels of *CD36*, *CPT1B*, *LCAD*, and *MCAD* were determined in C2C12 myotubes treated with DMSO, 50 μM, or 100 μM PIC for 24 hours. Results are shown as mean ± SEM (n = 3 - 5). *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$.

3.5. Knockdown of SIRT1 Suppressed the PIC-Induced Upregulation of Energy Metabolism-Related Gene Expression

Finally, to verify the hypothesis that SIRT1 regulates these signaling pathways, we performed SIRT1 knockdown using siRNA. Following the knockdown, the previously observed PIC-induced upregulation of both energy and lipid metabolism-related gene groups was significantly suppressed (Figure 6(A), Figure 6(B)). These results clearly demonstrate that SIRT1 plays a central role in controlling the PIC-induced enhancement of energy metabolism in skeletal muscle.



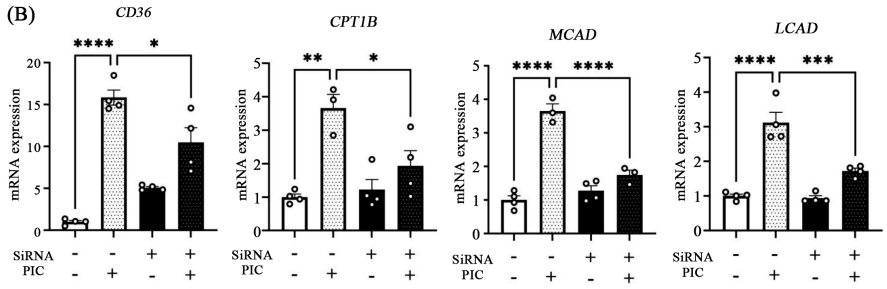


Figure 6. Knockdown of SIRT1 suppressed the PIC-induced upregulation of energy metabolism-related gene expression. (A) mRNA levels of *SIRT1*, *PDK4*, *PGC1A*, *PPARG* in C2C12 myotubes transfected with SIRT1 (+) or control (−) siRNA and then treated with DMSO, 50 μM, or 100 μM PIC for 24 hours. Results are presented as mean ± SEM (n = 4 - 5). **P* < 0.05, ***P* < 0.01, *****P* < 0.0001. (B) mRNA levels of *CD36*, *CPT1B*, *LCAD*, and *MCAD* in C2C12 myotubes transfected with SIRT1 (+) or control (−) siRNA and then treated with DMSO, 50 μM, or 100 μM PIC for 24 hours. Results are presented as mean ± SEM (n = 4 - 5). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

4. Discussion

In this study, PIC treatment increased intracellular NAD^+ levels and enhanced the expression of genes related to NAD^+ synthesis (**Figure 4**), indicating upregulated NAD^+ synthesis within the cell. NAD^+ plays a crucial role as a redox coenzyme in energy metabolism, generating NADH through proton reduction [29]. There are three main NAD^+ synthesis pathways: the kynurenine pathway, the Preiss-Handler pathway, and the salvage pathway. In skeletal muscle, the salvage pathway mediated by NAMPT maintains proper NAD^+ levels [30]. Muscle-specific NAMPT knockout mice show severe reductions in muscle NAD^+ levels, leading to decreased muscle energy and function, which can be restored by administering nicotinamide riboside (NR) [30]. Furthermore, total NAD^+ and ATP levels in skeletal muscle reportedly decline with aging [31]. Thus, NAD^+ *in vivo* influences metabolism, muscle function, and age-related changes. Supplementation with NR or nicotinamide mononucleotide (NMN), intermediates in NAD^+ synthesis, effectively restores NAD^+ levels [32]. Increased NAD^+ synthesis *in vivo* is induced by Slc12a8, promoting sodium-dependent NMN transport to maintain NAD^+ synthesis during aging. The PIC-induced increase in NAD^+ levels may also be mediated by elevated Slc12a8 expression. Future studies should investigate the systemic metabolic dynamics, from PIC uptake and metabolism to improvements in skeletal muscle function, rather than focusing solely on skeletal muscle.

Activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK) is involved in enhancing NAD synthesis. AMPK senses changes in the intracellular ATP to AMP ratio and regulates metabolism [33]. Activated AMPK inhibits the synthesis of glycogen, fatty acids, cholesterol, and proteins while promoting glucose uptake, fatty acid oxidation, mitochondrial metabolism, and autophagy [34]. AMPK activation is also reported to enhance NAD^+ synthesis through the salvage pathway, leading to SIRT1 activation [35]. In our study, the observed upregulation of NAD^+ synthesis pathway genes and increased SIRT1 expression sug-

gested possible involvement of AMPK. However, contrary to our expectations, we did not observe AMPK upregulation following PIC treatment (data not shown). One explanation may be that AMPK, as an early sensor of energy metabolic changes, was transiently activated at earlier time points. For example, Spears *et al.* reported that when C2C12 myotubes were treated with RES for just 1 hour, phosphorylated AMPK levels significantly increased [36]. Thus, AMPK activation might have occurred before the 24-hour time point used in our experiments; this represents a limitation of the current study. Another possibility is that AMPK and PGC1A independently interact with PPARB/D [37]. Since PIC treatment increased the expression of PPARB/D and PGC1A (**Figure 4** and **Figure 5**), it is possible that PIC-induced changes in metabolic gene expression occur via PGC1A alone, rather than through AMPK.

PIC is a stilbene-class polyphenol with four hydroxyl groups, and its antioxidant activity has been extensively studied. RES and trans-4-hydroxystilbene, also part of the stilbene group and found in wine, have strong antioxidant capacities. This study's novel finding is that PIC enhances skeletal muscle energy metabolism. The metabolic-enhancing effects of polyphenols have been previously reported. For example, RES, another stilbene compound, shifts carbohydrate metabolism toward lipid oxidation by increasing AMPK and inhibiting acetyl-CoA carboxylase, thereby reducing the whole-body respiratory quotient and promoting fatty acid synthesis in tissues and fatty acid β-oxidation in skeletal muscle [38].

Additionally, human clinical trials have been conducted using beverages rich in polyphenols such as anthocyanins. Consumption of polyphenol-rich beverages (including flavonoids, tannins, anthocyanins, and chlorogenic acid) resulted in decreased fat mass, increased lean mass, and elevated glucagon-like peptide-1 levels [39]. These reports suggest that various polyphenols can influence metabolic functions in the body. Administering beverages containing PIC for one week led to a significant increase in SIRT1 gene expression in the whole blood of participants, particularly postmenopausal women [21]. Moreover, subgroup analysis based on SIRT1 expression showed that PIC had a significant effect on older and higher-BMI populations. Thus, the increase in SIRT1 expression induced by PIC suggests that PIC could contribute to increased lipid utilization and the treatment of age-related diseases [21]. Furthermore, it has been reported that in nutritionally deprived skeletal muscle, oxidative stress induces AMPK-independent inhibition of SIRT1, leading to suppressed PGC1A expression downstream [40]. Such metabolic alterations are characteristic of muscle atrophy caused by malnutrition. Therefore, PIC may also contribute to the prevention of muscle atrophy under malnourished conditions through its ability to increase SIRT1 expression in skeletal muscle.

Future studies should investigate whether the PIC-induced enhancements of skeletal muscle metabolism observed in this study can translate into improvements in human conditions, such as reductions in body fat and the amelioration of muscle dysfunction (e.g. atrophy caused by oxidative stress).

5. Conclusion

In this study, we demonstrated that treating C2C12 myotubes with PIC increases SIRT1 activity and expression by elevating NAD^+ levels. Furthermore, SIRT1 activates downstream signaling pathways that upregulate energy metabolism-related genes, ultimately enhancing mitochondrial activity. These findings suggest that PIC may improve exercise performance and ameliorate systemic metabolic disorders by activating the energy state of skeletal muscle.

Authors' Contributions

Haruka Tsuda: Writing—original draft, data curation, formal analysis, investigation, methodology, and visualization; **Takayuki Uchida**: Conceptualization, funding acquisition, project administration, supervision, investigation, and writing—review & editing; **Saki Okuno**: Investigation; **Anayt Ulla**: Methodology; **Shinpei Kawakami**: Writing—review & editing, and resources; **Sadao Mori**: Methodology and resources; **Hiroko Maruki-Uchida**: Methodology and resources; **Takeshi Nikawa**: Resources and supervision.

Availability of Data and Materials

The data supporting these findings are available from the corresponding author upon reasonable request.

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Declaration

Shinpei Kawakami, Sadao Mori, and Hiroko Maruki-Uchida are employees of Morinaga & Co., Ltd.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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